

A novel electrochemical detection method for aptamer biosensors

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Received 11 November 2004; received in revised form 2 February 2005; accepted 4 February 2005

Available online 25 March 2005

Abstract

A beacon aptamer-based biosensor for the detection of thrombin was developed using electrochemical transduction method. Gold surface was modified with a beacon aptamer covalently linked at 5'-terminus with a linker containing a primary aliphatic amine. Methylene blue (MB) was intercalated into the beacon sequence, and used as an electrochemical marker. When the beacon aptamer immobilized on gold surface encounters thrombin, the hairpin forming beacon aptamer is conformationally changed to release the intercalated MB, resulting a decrease in electrical current intensity in voltamogram. The peak signal of the MB is clearly decreased by the binding of thrombin onto the beacon aptamer. The linear range of the signal was observed between 0 and 50.8 nM of thrombin with 0.999 correlation factor. This method was able to linearly and selectively detect thrombin with a detection limit of 11 nM.

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Keywords: Beacon aptamer; Biosensor; Self-assembled monolayer; Methylene blue; Electrochemistry

1. Introduction

Aptamers are artificial nucleic acid ligands showing specific binding affinity for amino acids, drugs, proteins and other small molecules, which can be screened through the systematic evolution of ligands by exponential enrichment (SELEX) process from random RNA or DNA libraries (Tuerk and Gold, 1990; Ellington and Szostak, 1990). They have potential applications as a recognition element in analytical and diagnostic assays, as aptamers can be easily screened, designed and evolved in vitro (Jayasena, 1999; James, 2000; Famulok et al., 2000; Hermann and Patel, 2000). Coupling the aptamer to an appropriate detection system offers the possibility of sensing analytes in solution. Although DNA aptamer is recently becoming quite popular due to its easiness in handling and reproduction, RNA aptamer, in general, has a few, but good advantages over DNA probes in designing molecules with high binding affinity and developing in vivo antisensor and riboswitch (Osborne and Ellington, 1997; Soukup and Breaker, 1999; Famulok, 1999; Winkler

and Breaker, 2003). The key issue in the development of aptamer-based analytical methods and sensors is to convert target recognition into a measurable signal. DNA (or RNA) beacon aptamer is a single-stranded oligonucleotide containing a short stem and a loop region (Tyagi and Kramer, 1996). The short stem structure having complementary sequence at both ends of the beacon aptamer can be used as a transducing element of the signal generated by the loop aptamer of high binding affinity to target molecules. Upon aptamer-target molecule binding, if the Gibb's free energy of the conformational changes of the loop region becomes greater than that of the binding energy of the stem region, the broken stem structure leads to changes in the vibrational energy of the fluorescent tagging or intercalating molecules at the stem structure.

Such beacon type aptamer-target binding reactions can be detected by several methods. Actually, various methods based on acoustic (Liss et al., 2002; Savran et al., 2004) and optical (Tyagi and Kramer, 1996; Potyrailo et al., 1998; Vet et al., 1999; Yamamoto and Kumar, 2000; Hamaguchi et al., 2001; Li et al., 2002; Culha et al., 2003) detection have been reported by immobilizing the aptamer on a solid surface. However, electrochemical transducer-based methods for

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the use of DNA (or RNA) beacon aptamer are rarely investigated compared with the methods based on optical transducers. If the beacon aptamer-target binding is detected by electrochemical transduction, it will become a very attractive tool for miniaturization and packaging. Among the various sensing devices developed so far, electrochemical method is, in general, superior to optical methods because of rapid response, simple and easy handling, and low cost (Palecek and Fojta, 1994; Wang, 1999; Wang, 2000).

Methylene blue (MB) belongs to phenothiazine family and MB is an aromatic cationic dye showing optically and electrochemically active properties. MB usually binds to DNA (Tuite and Norden, 1994; Kelley et al., 1999; Erdem et al., 2000; Rohs et al., 2000) and tRNA (Antony et al., 1995) via intercalation. It was reported that its intercalation into DNA or tRNA was mediated by the interaction with two guanine bases, suggesting that minimum two base pairs of G–C coupling in the stem structure of the beacon aptamer may lead to intercalation of MB (Tuite and Kelly, 1995; Rohs et al., 2000; Tuite and Kelley, 1993; Fusimoto et al., 1994; Tuite and Norden, 1994; Friedman and Brown, 1978).

In this paper, we introduce an electrochemical detection method of a beacon type aptamer biosensor using the intercalation of MB, where the MB is used as an electrochemical marker. Binding of thrombin, i.e. model protein is correlated with the changes in electrical current intensity in voltammetry. This approach might be able to provide a means to quantitative analysis of target proteins as well as any analytes whose aptamers can be selected, suggesting that this technique can be used to develop the aptamer beacon as an aptamer biosensor.

2. Experimental

2.1. Materials

Methylene blue·3H₂O (MB, 85% Sigma), 11-mercaptopundecanoic acid (MUA, 95% Aldrich), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, >98% Aldrich), potassium ferrocyanide (K₃[Fe(CN)₆], Sigma), urea (ACS reagent, Sigma) and *N*-hydroxysuccinimide (NHS, 97% Aldrich) were used without further purification. The beacon aptamer modified at the 5'-terminus with a primary aliphatic amine based linker were purchased from Bioneer Co. (Daejeon, Korea). Its sequence is as follows: 5'-NH₂-(CH₂)₆-TTC CAA CGG TTG GTG TGG TTG G-3'. The beacon aptamer stock solution (1.0 mM) was diluted with dH₂O and stored in the freezer before use. More dilute solutions of beacon aptamer were prepared with 25 mM Tris–HCl (pH 7.6) containing 5 mM MgCl₂ and 100 mM NaCl (*I* = 0.14 M). Thrombin (from human plasma) and BSA (Albumin Bovine 98.0%) were purchased from Sigma and aMResco, respectively. β-Casein (from bovine milk) and ovalbumin (chicken egg) were purchased from Sigma. Other chemicals were of analytical reagent grade. The deionized

water having 18 MΩ cm resistivity was used in making all the solutions.

2.2. Apparatus

UV–vis spectra were recorded on a Hewlett-Packard 8453 spectrophotometer. Fluorescence spectra were recorded on Jasco FP-777 spectrofluorometer. Cell with a 1.0 cm path length were used for absorbance and fluorescence measurements. Electrochemical measurements were performed using CHI 660A electrochemical analyzer. A three electrode configuration consisting of a modified gold working electrode (area = 0.28 cm²), an Ag|AgCl|KCl (sat'd) reference electrode, and a platinum wire auxiliary electrode was used. Gold (Au) films of 200 nm thick over Ti/glass were kindly provided by Prof. Kim, Yong-Kwon, in school of electrical engineering and computer science at Seoul National University. The experimental temperature was controlled at 25 ± 2 °C. All measurements were carried out in completely deaerated solution. All measurements were performed in 1 mL of 25 mM Tris–HCl (pH 7.6) containing 5 mM MgCl₂ and 100 mM NaCl (*I* = 0.14 M) as background electrolyte.

2.3. Preparation of beacon aptamer surfaces

Immobilization of the beacon aptamer and target binding processes are illustrated in Fig. 1. Before chemisorption, the gold film was cleaned with piranha solution (98% H₂SO₄:30% H₂O₂ = 70:30, v/v). The gold film was immersed in a freshly prepared piranha solution for 10 min, followed by washing twice with distilled water, and ultrasonically cleaning with ethanol and distilled water for 3 min, and finally drying with a stream of nitrogen gas before exposure to MUA solution. The MUA self-assembled monolayer (SAM) was prepared by soaking the clean gold film in freshly made 3:1 (v/v) EtOH:H₂O solution containing 20 mM 11-MUA for 18 h, followed by thorough rinsing with 3:1 (v/v) EtOH:H₂O and H₂O and subsequent drying with a stream of nitrogen gas. The clean MUA film was immersed in 50 mM phosphate buffer solution (pH 7.4) containing 2 mM EDC and 5 mM NHS to activate the carboxyl-terminated surface for 1 h, followed by rinsing with distilled water and ethanol, and drying with a stream of nitrogen gas. The activated 11-MUA film was rinsed with the 50 mM phosphate buffer and then immersed in 25 mM Tris–HCl buffer solution (pH 7.6, *I* = 0.14 M) containing 0.4 μM amine terminated beacon aptamer at room temperature for 2 h. The beacon aptamer film was rinsed thoroughly 25 mM Tris–HCl buffer (*I* = 0.14 M, pH 7.6) and soaked in 1% BSA solution to block direct contact between MB or thrombin and gold surface for 30 min. The blocked beacon aptamer film was soaked in 25 mM Tris–HCl buffer (pH 7.6, *I* = 0.14 M) to remove unbound BSA for 10 min and rinsed thoroughly with 25 mM Tris–HCl buffer. Thus, a beacon aptamer probe-modified gold electrode was obtained.

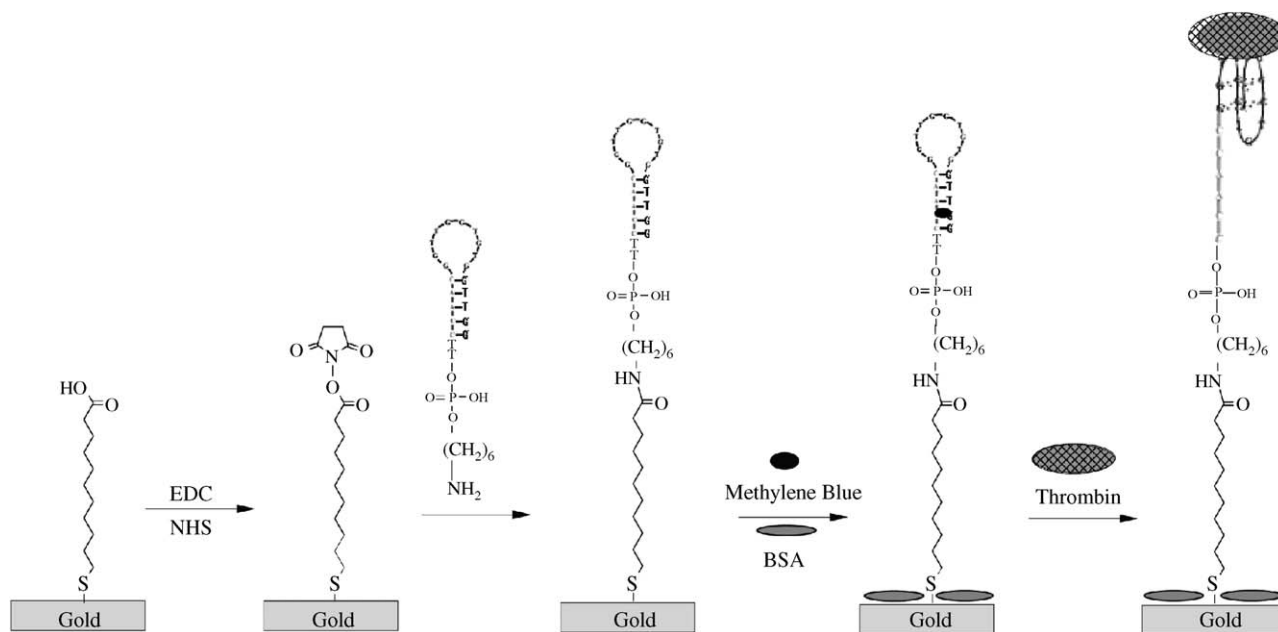


Fig. 1. Schematic representation of the immobilization of beacon aptamer and detection of its target protein.

2.4. Label binding to the beacon aptamer and electrochemical measurements

Methylene blue was accumulated on the beacon aptamer gold film by soaking in 25 mM Tris–HCl buffer (pH 7.6, $I=0.14$ M) containing 20 μM MB for 5 min without applying any potential. After that, the aptamer electrode was rinsed with 25 mM Tris–HCl buffer (pH 7.6) and was used in electrochemical measurement immediately.

2.5. Application to thrombin detection using electrochemical measurements

After accumulation of MB, the beacon aptamer electrodes were immersed in 25 mM Tris–HCl buffer (pH 7.6, $I=0.14$ M) containing 400 nM thrombin at room temperature for 30 min. The electrodes were rinsed with 25 mM Tris–HCl buffer (pH 7.6) and then drying with a stream of nitrogen gas. Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were performed on the fresh electrodes. The electrochemical signals were obtained by using DPV with amplitude of 10 mV at 20 mV s^{-1} scan rate and CV at 50 mV s^{-1} scan rate in 25 mM Tris–HCl buffer (pH 7.6, $I=0.14$ M), respectively.

2.6. Stability and regeneration of the beacon aptamer probe

To examine temperature stability of the beacon aptamer probe, the beacon aptamer probe was stored in 25 mM Tris–HCl buffer solution at different temperatures (i.e. 4 °C, room temperature, and 37 °C, respectively) in time (i.e. 1, 3, 5 and 9 days). Its temperature stability was examined

in 25 mM Tris–HCl buffer solution by DPV. To assure the detection of any lost amount of the beacon aptamer during the experiments, the changes in the signal from the probe were compared before and after the reannealing procedure.

To examine regeneration of the beacon aptamer probe, the beacon aptamer electrode was rinsed with 7 M urea solution and 25 mM Tris–HCl buffer solution two times, respectively. The beacon aptamer was subsequently heated to 70 °C for 3 min, and allowed to cool to room temperature or 4 °C for 15 min or 30 min, respectively. After the cooling step, the beacon aptamer was incubated with 20 μM MB solution for 3 min, and subsequently washed. Using DPV, the signal change caused by the intercalated MB was observed before and after target binding. The measurements were repeated five times.

3. Results and discussion

3.1. Intercalation of MB in the beacon aptamer

Fig. 2(A) shows UV–vis spectra of MB with increases in the amount of beacon aptamer in 25 mM Tris–HCl buffer (pH 7.6, $I=0.14$ M), where the concentration of MB was fixed at 1.57 μM . They displayed two peaks around 250, 665 nm, respectively, which corresponds to the maximum peak of MB, i.e. 665 nm. Upon addition of the beacon aptamer to MB solution, the intensity of the peak decreased with red shift. These bathochromic (red shift) and hypochromic (intensity decrease) phenomena can be explained by the intercalation of MB to the beacon aptamer. The result was similar to that of the previous reports (Antony et al., 1995). As MB is a weak fluorophore, its quenching efficiency would change

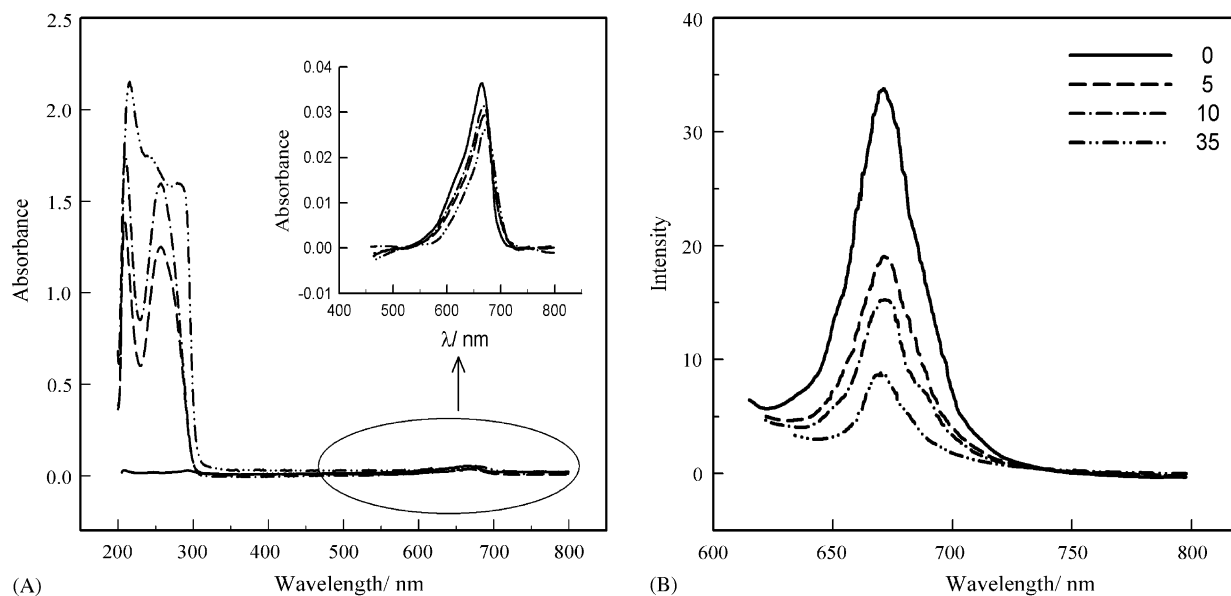


Fig. 2. (A) UV-vis spectra and (B) fluorescence spectra of MB with increases in the amount of beacon aptamer. The concentration of MB was fixed at 1.52 μM . The molar ratio of thrombin beacon aptamer to MB varied from 0, 5, 10, to 35. $\lambda_{\text{max}}^{\text{em}} = 600 \text{ nm}$.

according to its binding mode. When MB is intercalated into DNA, its quenching efficiency would increase owing to the environmental effect, resulting concomitant decrease in fluorescence intensity. The fluorescence spectra were recorded at excitation 600 nm under the same condition as that in UV-vis experiment (Fig. 2(B)). Fig. 2(B) shows that the fluorescence intensity decreases as we increase the concentration of the beacon aptamer in the solution, suggesting that free MB in the solution participate in the intercalation. Another explanation would be MB dimerization and aggregation. MB is known to naturally dimerize and aggregate under certain conditions (Bergmann and O'Konski, 1963). It is reported that the dimerization and aggregation of dye molecules are related to a decrease in fluorescence emission intensity, suggesting that free MB in the solution might dimerize and aggregate to a certain extent (Thomas et al., 1995). The above results agree well with the previously reported data, saying that MB mainly binds to the beacon aptamer via intercalation.

3.2. Electrochemical responses of intercalated MB on beacon aptamer probe

First of all, to demonstrate again that such beacon aptamer reaction with MB or other RNA/DNA modifying redox-reagents can be detected by electrical method, the electrochemical responses to the similar reactions were measured by CV. To establish modified gold surface with beacon aptamer, cyclic voltammograms of $[\text{Fe}(\text{CN})_6]^{3-}$ on bare and the modified beacon aptamer gold electrodes in 25 mM Tris-HCl buffer (pH 7.6, $I = 0.14 \text{ M}$) containing 0.1 mM $[\text{Fe}(\text{CN})_6]^{3-}$ were generated by scanning from +0.5 V to -0.1 V at 100 mV s^{-1} (Fig. 3(A)).

Fig. 3(A) shows that the redox peaks of $[\text{Fe}(\text{CN})_6]^{3-}$ were only detected at the bare gold electrode. As both

the backbone phosphate group of the beacon aptamer and $[\text{Fe}(\text{CN})_6]^{3-}$ have negative charges, it is likely that electrostatic repulsive interactions between the phosphate group of the modified electrode and $[\text{Fe}(\text{CN})_6]^{3-}$ dominate, and $[\text{Fe}(\text{CN})_6]^{3-}$ would not bind or intercalate into the beacon aptamer probe. In contrast, when the electrode modified with the beacon aptamer incubated with 20 μM MB solution was transferred to the blank buffer solution, i.e. 25 mM Tris-HCl buffer (pH 7.6, $I = 0.14 \text{ M}$), (Fig. 3(B)) cyclic voltammogram was obtained. The cathodic peak currents increased quite linearly ($R^2 = 0.999$) with increasing scan rate, indicating a typical characteristic curve of immobilized species (Bard and Faulkner, 2001; Abruna, 1988). Fig. 3 strongly supports the fact that the beacon aptamer is immobilized on the gold surface of the electrode, and MB is also properly intercalated to the modified beacon aptamer. To avoid the high electrostatic attractions between the positively charged MB and the phosphate group of the beacon aptamer probe, all the electrochemical measurements were performed under a high ionic strength condition, i.e. $I = 0.14 \text{ M}$ (Kara et al., 2002). The peak potential of the intercalated MB showed a constant value at over 0.05 M ionic strength (data not shown). Therefore, it was reasonable to assume that the ionic strength used in this experiment is high enough to shield the electrostatic binding of MB to negatively charged aptamer probe. In addition, to reduce non-specific MB binding to the electrode surface, the gold electrode surface was treated with BSA prior to the addition of the redox reagents such as MB. It was also confirmed that MB does not greatly bind to the BSA-treated gold surface, but does negligibly bind (Fig. 4). Therefore, we could confirm that the electrochemical signal of the cyclic voltammogram in Fig. 3(B) was mainly resulted from the intercalated MB.

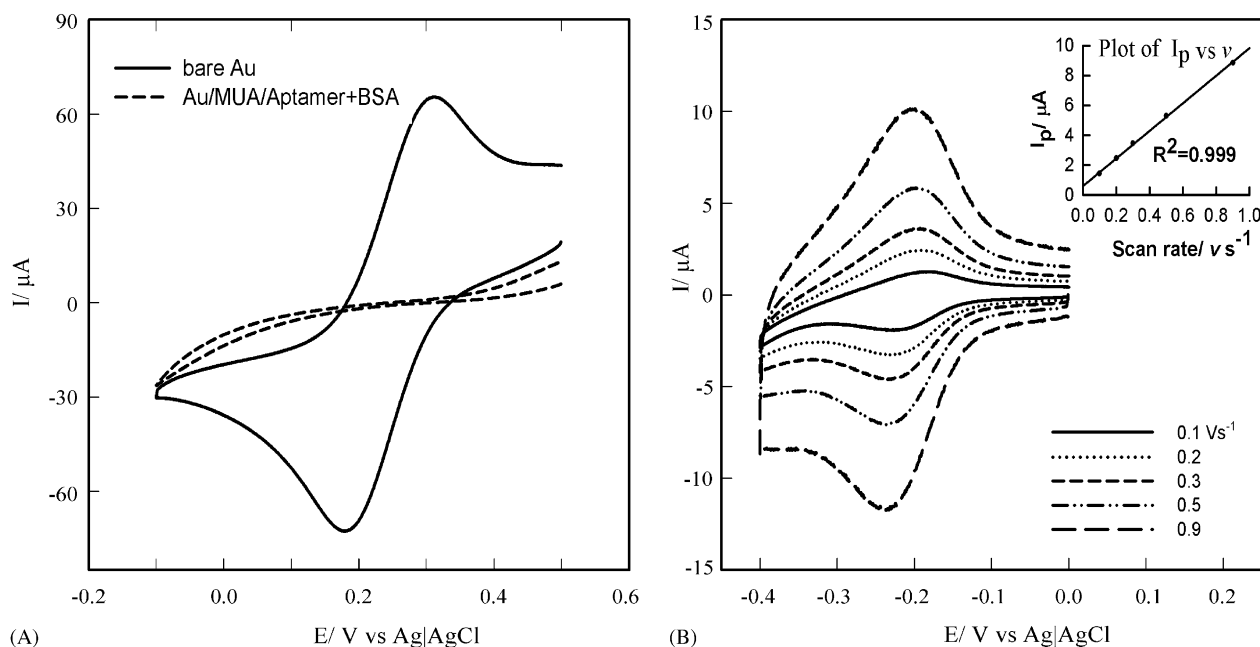


Fig. 3. (A) Cyclic voltammograms for 0.1 mM $[\text{Fe}(\text{CN})_6]^{3-}$ in 25 mM Tris-HCl buffer (pH 7.6, $I=0.14$ M) at bare (solid line) and the modified beacon aptamer gold electrodes (dashed line). Scan rate = 100 mV s^{-1} . (B) Cyclic voltammograms after incubation of $20 \mu\text{M}$ MB in 25 mM Tris-HCl buffer (pH 7.6, $I=0.14$ M) at the modified beacon aptamer gold electrodes with increasing scan rate (inset: plot of peak current vs. scan rate. Electrode area = 0.28 cm^2).

Fig. 4 shows a differential pulse voltammograms (DPVs) on the modified beacon aptamer electrode (solid line) and MUA electrode (dashed line) treated with $20 \mu\text{M}$ MB in 25 mM Tris-HCl buffer. Reduction of the MB usually proceeds via two electrons and one proton transfers, and forms leucomethylene blue. The potential value of the reduction peak of the intercalated MB was -197 mV which results cathodic shift of about -25 mV relative to the value in aqueous solution. ΔE_{fwhm} value was about 164 mV for the reduction

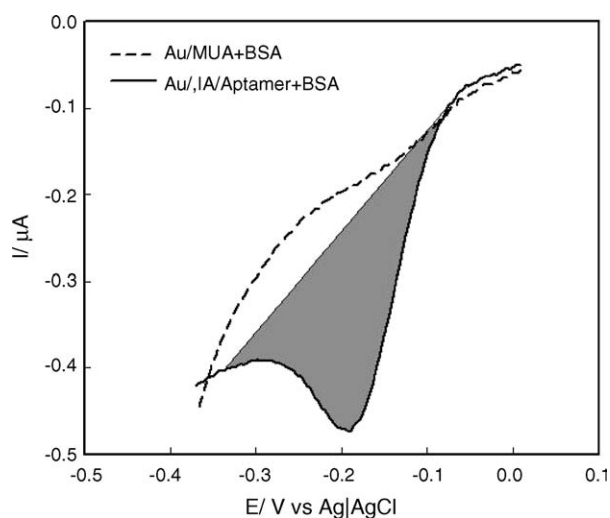


Fig. 4. Differential pulse voltammograms for the incorporated MB on the modified MUA electrode (dashed line) and the modified beacon aptamer electrode (solid line) after the treatment of 1% BSA in 25 mM Tris-HCl buffer (pH 7.6, $I=0.14$ M), respectively. Amplitude = 10 mV , pulse width = 0.05 s . Electrode area = 0.28 cm^2 .

processes. This value is much larger than the theoretical value of $90.6/n$ (mV) for a $2e^-$ Nernstian system, where n is the mole number of electrons transferred, indicating the presence of lateral interaction and a higher degree of organization in the monolayer (Bard and Faulkner, 2001). The broadening of peak is related with environmental effect of monolayer. After BSA treatment to block between MB and Au surface, the peak of MB is not shown on the modified MUA electrode without the beacon aptamer. Therefore, as the modified MUA surface is blocked with BSA, MB cannot interact with the BSA on the surface. It can be explained as repulsion between negatively charged BSA and neutral MB. Thus, it is understood that there are a higher degree of repulsion (Bang and Jeon, 2001) and different environment of reduction potential.

To quantitatively understand the coverage of beacon aptamers on the gold electrode surface, the surface coverage of MB is estimated by integrating the voltammogram. We repeated the same experiment five times to observe its reproducibility. The estimated value was $23 (\pm 2) \text{ pmol cm}^{-2}$. As the current density depends on the MB intercalated into the beacon aptamer, the surface coverage of beacon aptamer can be determined by assuming that all the beacon aptamers on the gold surface are saturated with the intercalated MB. The calculated value is 2.5 times lower than the reported one forming a full monolayer of 15-mer double stranded DNA (Kelley and Barton, 1997; Kelley et al., 1998). Through this calculation, the occupied area of a beacon aptamer per one molecule on the gold surface is estimated to be larger than that of 15-mer double strand DNA.

To examine the stability of the beacon aptamer probe, we compared its temperature stability by incubating the beacon

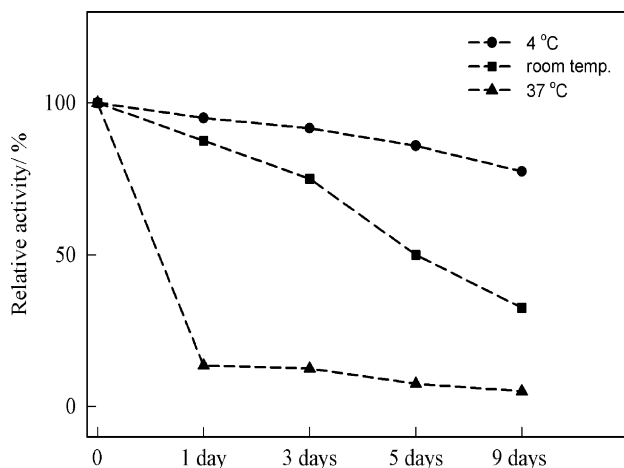
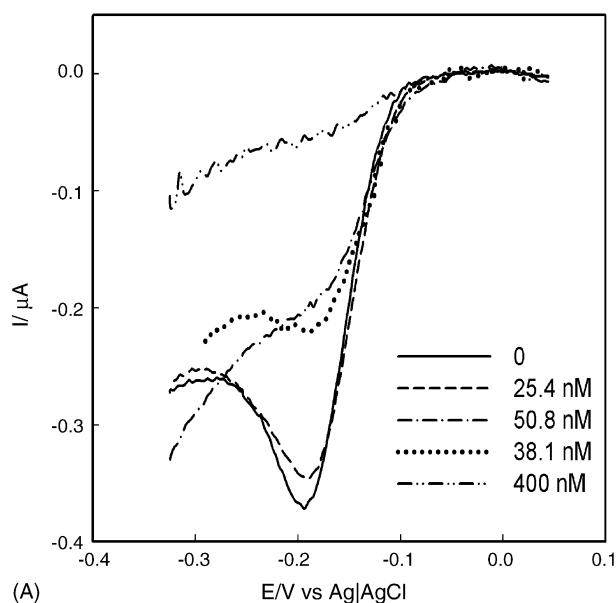


Fig. 5. The stability of the beacon aptamer probe at different temperatures: (●) 4 °C, (■) room temperature and (▲) 37 °C. The relative activity was calculated from the surface coverage of the functional beacon aptamer on the electrode surface measured in the following condition: electrode area = 0.28 cm², cell volume = 1 mL of 25 mM Tris–HCl buffer (pH 7.6, $I = 0.14$ M), amplitude and pulse width in DPV were 10 mV and 0.05 s, respectively.

aptamer electrode in 25 mM Tris–HCl buffer (pH 7.6, $I = 0.14$ M) at different temperatures. (Fig. 5) The relative activity indicates the relative surface coverage value of the functional beacon aptamers, assuming the initial surface coverage of 23 (± 2) pmol cm⁻² as 100% of a full monolayer. At 4 °C, the relative activity decreased rather slowly with time, suggesting that the beacon aptamer probe should be stored at this temperature before usage. However, the activity decreased rapidly at room temperature and 37 °C. At 37 °C, only 15% of its original activity was remained in a day.



In addition, to examine the repeated use of the beacon aptamer probe, the beacon aptamer electrode was rinsed with 7 M urea solution, and annealed at 70 °C for 3 min. After the treatment, the beacon aptamer was incubated with 20 μM MB solution and subsequently washed, and the signal change of the intercalated MB was observed before and after target binding. We repeated the same experiment five times. Then, till the fourth time, 90% of its original activity was recovered, and clear signal changes could be observed. Regardless of the different incubation temperatures and sampling time points in time, the recovery of 90% of its original activity of the probes was always the same after the annealing procedure, indicating that although the beacon aptamer probe is easily deactivated under different storage conditions, the probe can be simply and functionally reactivated.

3.3. Electrochemical responses of the interaction between beacon aptamer probe and target protein

To identify any changes in the voltammogram upon target binding, thrombin target samples were challenged to the beacon aptamer electrode. After the binding thrombin samples to the beacon aptamer electrode, the electrode was rinsed with 25 mM Tris buffer and the electrode was transferred into the blank Tris buffer solution. The electrochemical responses are shown in Fig. 6. The current signals in Fig. 6(A) were logged and compared after normalization of the baseline. The peak signals of MB decreased with increases in the concentration of thrombin. According to the literature (Hamaguchi et al., 2001), thrombin is expected to be bound to both loop regions and a part of the stem structure of the beacon aptamer. When the beacon aptamer on the gold electrode surface encounters

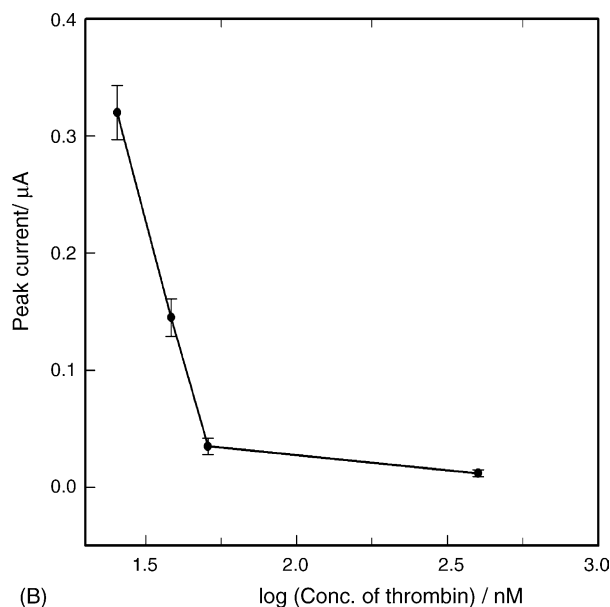


Fig. 6. (A) Differential pulse voltammograms for intercalated MB on the modified beacon aptamer electrode with increasing concentration of thrombin in 25 mM Tris–HCl buffer (pH 7.6, $I = 0.14$ M). Amplitude = 10 mV, Pulse width = 0.05 s. Electrode area = 0.28 cm². (B) Plot of peak current vs. log (concentration of thrombin) for (A).

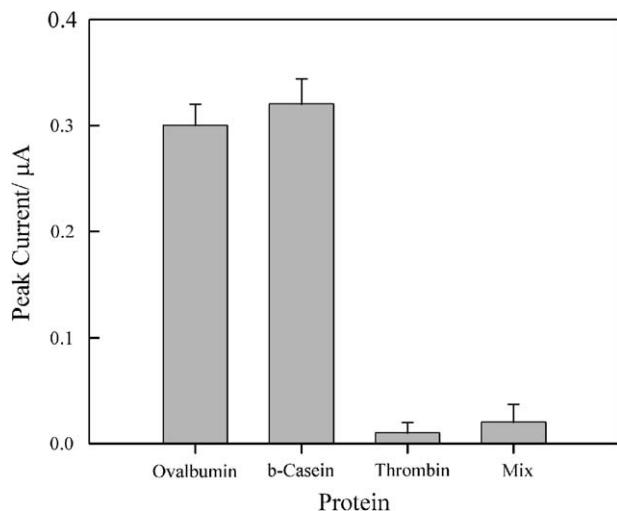


Fig. 7. Bar chart of DPV responses for intercalated MB in control experiments. MB peak current obtained after exposure to each protein (400 nM each) and a mixture of three protein solutions using the modified beacon aptamer gold electrodes with intercalated MB.

the thrombin, the hairpin forming beacon aptamer is expected to be broken by subsequent conformational changes in the structure of the beacon aptamer. Then, as we increase the sample thrombin concentration, the peak signal of the MB would be affected by the reduction in the concentration of intercalated MB in the beacon aptamer. When the concentration of thrombin is increased from 25.4 nM till 400 nM, the current signal of MB gradually decreased. The stoichiometric molar ratios of beacon aptamer to target corresponding to 23 (± 2) pmol cm⁻² of the surface coverage are 0.5, 0.7, 1 and 8, respectively. Until the concentration of thrombin is 50.8 nM, the decrease in current signal is clearly visible. And the peak signal of the intercalated MB almost disappeared at over 50.8 nM. Considering these results, the detection was made at over 25.4 nM target thrombin on the beacon aptamer probe. The best discriminating ability for target thrombin was observed when 50.8 nM (i.e. molar ratios of beacon aptamer to target = 1:1) target thrombin was encountered to the beacon aptamer on the gold electrode surface. A linear range can be observed between 0 and 50.8 nM with correlation factor 0.999. The regression equation is $y = -0.98 \log x + 1.65$ (here, x is the concentration of target protein (nM) and y , the peak current of MB (μA)). The estimated detection limit of the target protein was 11 nM.

To confirm the binding specificity of the beacon aptamer sensor to the target molecule, control experiments were performed using 20 μM MB and 400 nM of beta-casein and ovalbumin, respectively (Fig. 7). Among them, only the thrombin sample showed the decrease in peak current, suggesting that the beacon aptamer sensor is quite specific to the target molecule. We repeated the experiment three times to observe its reproducibility, and had a consistent data as shown in the error bar in Fig. 7. We also examined cross sensitivity of the beacon aptamer probe in a mixture of three different proteins containing thrombin. The signal obtained from the

complex mixture was similar to that obtained from only thrombin.

4. Conclusion

The interaction of thrombin with beacon aptamer immobilized on gold was investigated by using electrochemical methods. MB can be intercalated into the beacon aptamer and can be used as an electrochemical marker for target protein binding on the beacon aptamer electrode. The peak currents of MB start to decrease by the addition of thrombin. The cathodic peak current of MB has a linear relationship with the logarithmic value of thrombin concentration ranging from 0 to 50.8 nM. The estimated detection limit of the target thrombin was 11 nM. The results demonstrate that the electrochemical method using aptamer probe is convenient, and allows quantitative detection of target protein. It also shows a possibility that an electrochemical tool can be used for the aptamer sensor displaying high binding affinity and specificity for bioanalytical instrument.

Acknowledgements

We thank Prof. Kim, Yong-Kwon and Mr. Min-Soo Kim in School of Electrical Engineering and Computer science, Seoul National University for providing gold films. This project was partially supported by Advanced Backbone IT Technology Development Project (IMT2000-B3-2) of the Ministry of Information and Communication.

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